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13. ABSTRACT (Maximum 200 words)
Cortical collecting duct cells were cultured on permeable membranes and used to assess cellular transport properties for calcium/cadmium. Functional viability of the cells in culture were affirmed by their ability to absorb sodium and chloride, secrete protons and potassium, and respond appropriately to the hormones vassopressin and aldosterone. Calcium absorption by these cells was affected neither by sodium blockers or hormone inducers of cyclic AMP nor, surprisingly, by various calcium channel blockers. However, when the pH of the culture medium on the apical side of the cells was controlled artificially by buffer, calcium absorption was found to be affected. Decreasing the pH inhibited calcium uptake by cells, indicating that calcium and H+ transport were linked, possibly by a mechanism involving a H+/Ca++ exchanger. Cadmium had no apparent effect on calcium absorption untill cadmium concentrations reached levels at which nonspecific, cytotoxic effects were observed. In another separate project, initial research efforts were undertaken to use electron paramagnetic resonance (EPR) to explain the mechanism by which endothelial/smooth muscle cells release the putative endothelium-derived relaxing factor (EDRF), nitric oxide (NO), from vasodilator drugs. EPR detection on NO was accomplished when the model drug nitroprusside was chemically reduced in a cell-

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free system. However, detection of NO was not possible by EPR when nitroprusside was added to a responsive cellular system. Alternative method modifications have been explored to enable detection by EPR trapping agent for NO. Further testing of these modifications are continuing (beyond the period of funding by tha AFOSR grant) in Dr. Roger Smith's laboratory at Dartmouth Medical School.

Rochelle: Progress Report for July 1, 1990 through June 30, 1992

The 2 years of funding by the AFOSR fellowship encompassed work on two research projects. The project for which the proposal was written ie. "Regulation of cadmium uptake in renal tubules" resulted in the production of one manuscript currently being reviewed by Kidney International and two abstracts. More recently, preliminary work on a project investigating mechanisms of nitric oxide production by endothelial and smooth muscle cells has resulted in two manuscripts and one abstract. The results of both projects are summarized below.

Calcium/cadmium transport by renal cortical collecting duct (CCD) cells

The progress report for July 1, 1990 through June 30, 1991 summarizes data from calcium transport experiments which have been incorporated in the manuscript "Active calcium absorption in primary cultures of cortical collecting duct cells". In this portion of the study, we provided evidence that CCD cells absorb calcium via active transport mechanisms and that a Na^+/H^+ exchange mechanism could be involved. CCD cells isolated and cultured for this study retained characteristics of CCD cells in vivo including absorption of sodium and chloride, secretion of potassium and protons (1,2,3) and responsiveness to the hormones aldosterone and vasopressin (3,4). These cells, when cultured on a permeable filter, formed a tight monolayer as demonstrated by the high transepithelial voltages (60 - 70 mV) and resistances (greater than 1000 ohm / cm^2) obtained. Net calcium transport was in the apical to basolateral direction (absorption) and resulted in accumulation of basolateral calcium to produce a basolateral/ apical calcium gradient of 120 after 24 hr. By adding amiloride to the apical side or ouabain to the basolateral side, we could demonstrate that calcium transport was not directly linked to sodium transport. Calcium absorption could be enhanced significantly with addition of cyclic AMP. This result suggested that calcium absorption could be hormonally regulated. Parathyroid hormone and antidiuretic hormone stimulate the production of cyclic AMP in the tubule segments connecting tubule and cortical collecting duct respectively (5). Niether of these hormones, however, stimulated calcium absorption within the 4 hr timecourse. The prototypical inorganic calcium channel blockers, nickel and lanthanum, also had no effect on net calcium transport and cadmium inhibited only at concentrations (50 and 100 μM) high enough to elicit nonspecific and perhaps general toxic effects.

During the next funded year, other calcium channel blockers were tested. We chose the organic compounds diltiazem, D-600 (an analog of verapimil), and nifedipine at concentrations 10 to 100 times concentrations demonstrated to block isolated calcium channels in patch clamp studies. These compounds block L-type channels, the type most commonly found in tissues outside of the nervous system. In our study, none of the compounds had any effects on calcium absorption. Gadolinium chloride, a compound shown to block a more recently characterized type of stretch-activated channel found in some renal tubule segments (6), also had no effect. Through the use of these calcium channel blockers, we found no evidence for calcium channels contributing to calcium absorption in CCD cells.

We did, however, find a parameter that had a reproducible effect on calcium absorption. CCD cells in culture secrete H^+ just as they do in situ. Proton secretion by the cultured cells results in significant acidification of the apical medium from pH 7.4 to pH 5.2 within 24 hr. To test the effects of pH, we adjusted the apical medium to a specific pH at the beginning of each experiment and added MES to buffer H^+ secreted, thereby stabilizing the pH throughout the 4 hr timecourse. We tested pH 4.0 through 8.0 in 0.5 increments as shown on the x axis of Figure 1. The rate of calcium flux or absorption, graphed on the y-axis, directly correlated with the pH ($r=0.94$) with decreasing pH inhibiting calcium absorption. These results indicate that calcium and H+ transport are linked. The graded response or what appears to be a kinetic inhibition suggests that the

mechanism could be a H^+/Ca^{2+} exchanger. This mechanism could conclevably be associated with the physiologic inhibition of calcium reabsorption by acidosis.

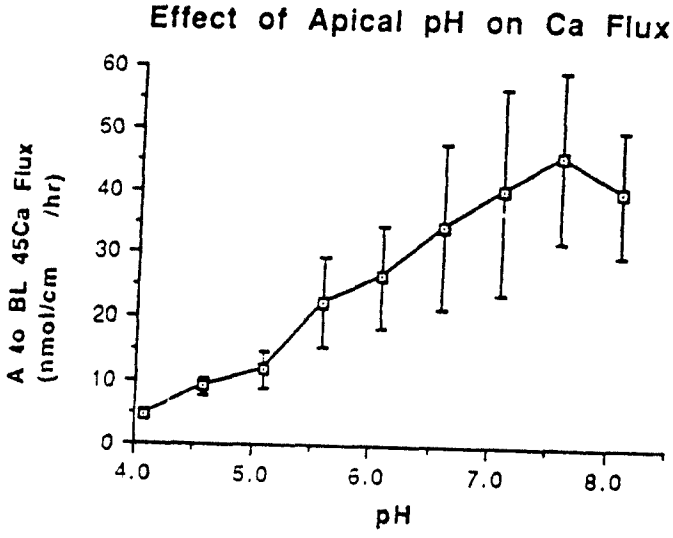

Figure 1

Nitric oxide (NO) production by endothelial and smooth muscle cells

The aim of this project is to determine under what conditions and by what mechanisms NO is released from certain vasodilator drugs by endothelial and/or smooth muscle cells. Many vasodilator drugs with an NO_x group in the molecular structure are presumed to act by release of the NO to the smooth muscle. Mechanisms of NO release by cells and definitive proof that NO is the biologically active molecule have not been clearly identified. During the period 12/10/91 through 6/30/92 significant preliminary work on methods for culturing cells, exposing cells to NO-vasodilator drugs, trapping products in the cell medium and detecting NO was accomplished.

The method we have been using to detect NO is electron paramagnetic resonance (EPR) because certain NO-bound molecules are readily visible by this method. The NO-releasing drug we have been working with is sodium nitroprusside (Fig.2). This drug releases NO after chemical reduction, and the reduced intermediates are EPR detectable (7).

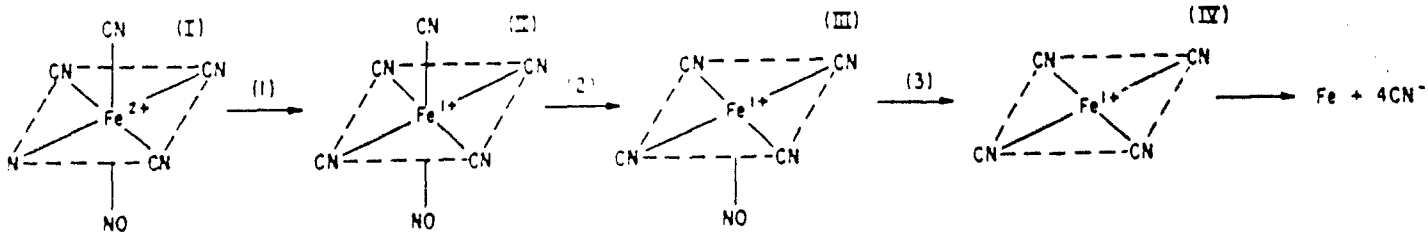

Figure 2

The first step was to define conditions under which the intermediates would be formed and detected. Nitroprusside was reduced with borohydride in aqueous solution and the intermediates were detectable for 10 min at room temperature. We could reproducibly interchange the intermediates by alternately freezing and thawing the sample. At room temperature the dashed line

spectrum and at -126^0 C the solid line spectrum could be obtained (Fig 3). These spectra have been demonstrated to correspond to intermediates III and II respectively (7) in Fig 2. Cyanide competitively inhibits the release of NO from reduced nitroprusside by preventing the release of the trans-cyanide (7). We could demonstrate that addition of equimolar cyanide to the nitroprusside solution above inhibited the formation of intermediate III and favored the formation of intermediate II.

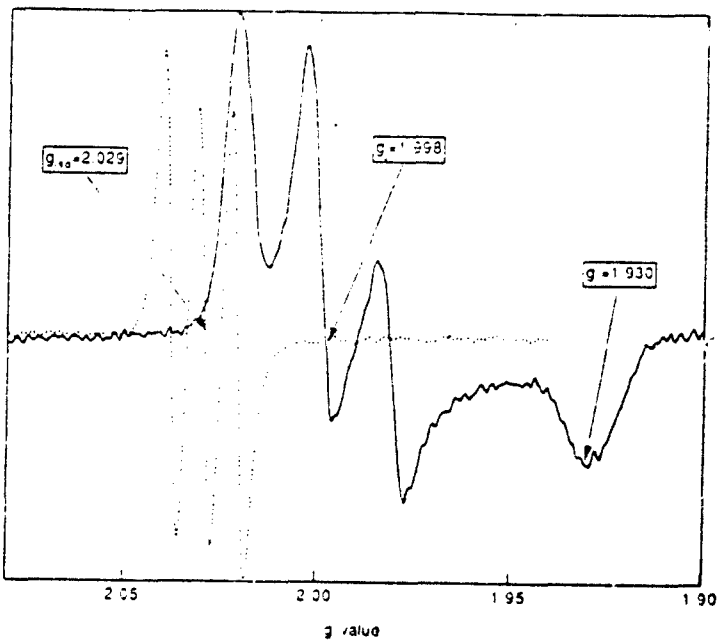

Figure 3

Techniques were perfected for separating and culturing endothelial and smooth muscle cells from porcine aortas. To answer the question if either of these cell types alone or in combination could perform the reduction and eventual release of NO, we have been experimenting with methods for trapping and detecting NO in the cell medium. In our first experiments, nitroprusside was added to the medium above monolayers of cells. Samples of medium were collected and scanned in the EPR spectrometer for the presence of nitroprusside intermediates. No EPR-detectable products were found. We have been investigating the following reasons for the lack of products: 1.) loss of the machinery for the NO-release pathway in the cultured cells, 2.) lack of sensitivity of the detection method 3.) instability of NO or nitroprusside intermediates in an oxygenated medium or 4.) the lack of reaction of nitroprusside with the cells.

In order to test the intactness of an NO production pathway, we measured the bradykinin-stimulated release of arachidonic acid. When stimulated with bradykinin, endothelial cells release arachidonic acid concurrently with and by a similar pathway as NO (8). We could demonstrate stimulated release of arachidonic acid with bradykinin, therefore, the cells probably contain the necessary responsive elements for NO release.

Another detection method was employed to find a more sensitive assay for NO release. The accumulation of free cyanide in the medium was analyzed as a measure of the reduction of nitroprusside. Samples collected at 2 and 30 min did not contain any detectable cyanide. There was a slight amount of cyanide detected in the 60 min samples but the values (2 x the blanks) were too close to the level of detection to confirm.

A technique for concentrating released NO in the cell medium is to collect samples eluted from a column of cells grown on beads. Endothelial cells seem to produce very small quantities of NO when stimulated (12). By growing cells on small beads, it is possible to maximize the amount of cells exposed to a certain volume and therefore concentrate the reaction products. We are currently adapting this technique to our experimental protocol.

In order to trap and thereby stabilize the NO in solution we have added hemoglobin to the medium. Hemoglobin readily binds NO but also directly reacts with nitroprusside (9, 10). Therefore, we carboxylated the hemoglobin, protecting it from reacting directly with the nitroprusside but not from binding free NO (11). Again, there were no EPR-detectable products within 4 min. We are currently working out techniques for accumulating samples for longer periods of time and concentrating the samples.

It is possible to produce significant vasodilation as well as HbNO from nitroprusside administered to the whole animal. It is also possible with cultured cells to indirectly measure the release of NO by stimulation of guanylate cyclase or smooth muscle strip relaxation. The EPR technique which we are adapting offers an advantage over these techniques of directly measuring the products from nitroprusside with cultured cells.

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